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Thermodynamic Partitioning Model for Hydrophobic Binding of Polypeptides by GroEL

II.† GroEL Recognizes Thermally Unfolded Mature β-lactamase

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By thermal equilibrium measurements we found a three-state folding behavior of mature *Escherichia coli* β-lactamase TEM2. The thermodynamically stable intermediate H had no enzymatic activity, but a native-like secondary structure. State H was 9 kcal mol^{-1} less stable than the native state N and 4 kcal mol^{-1} more stable than the totally unfolded state U, which is consistent with urea equilibrium measurements of mature β-lactamase measured under similar conditions.

Between 38°C and 50°C there was a decrease in the apparent equilibrium constant for dissociation K'_D of the complex between GroEL and mature β-lactamase, at least partially caused by a decrease in the thermodynamic stability of the native form of mature β-lactamase. GroEL-bound β-lactamase was released either after addition of ATP, or in the presence of a competing substrate (i.e. a single-chain antibody), or after lowering the temperature. Whereas at 10°C the folding reaction of mature β-lactamase was rate limiting, at 37°C the release reaction was the rate-determining step for the regain of β-lactamase activity, consistent with a decrease of the equilibrium constant for dissociation K_D of the complex with temperature. A temperature dependent behavior of GroEL was also observed, when measuring the anilinonaphthalene sulfonic acid (ANS) fluorescence of the chaperone. Similar to all other substrate proteins studied so far, the maximal tryptophan fluorescence of GroEL-bound β-lactamase was observed at 342 nm. Our results are compatible with a hydrophobic binding pocket of GroEL and confirm the suggested thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL.

Keywords: protein folding; molecular chaperone; signal sequence; molten globule

1. Introduction

This paper is the second of two on the interaction between the molecular chaperone GroEL and *Escherichia coli* β-lactamase TEM2, and the interaction with the mature enzyme is examined here. In the accompanying paper (Zahn *et al.*, 1994*a*) we compared the urea equilibrium and kinetic folding of precursor and mature β-lactamase, and their binding to GroEL at constant temperature (25°C). We showed that the reason for the higher affinity of GroEL for pre-β-lactamase, compared with mature β-lactamase, lies in the relatively low thermodynamic stability of pre-β-lactamase and a direct recognition of the hydrophobic signal sequence by GroEL.

From these results we suggested a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL. Whether a protein interacts with GroEL depends on the thermodynamic stability and the hydrophobicity of the substrate protein. The thermodynamic stability is ultimately a measure of the conditions under which hydrophobic residues become accessible. Thus there are two relevant equilibria, first the partitioning of the protein between a native state and an intermediate, and second the stability of the complex between GroEL and the protein in its intermediate form. The main criterion for binding was proposed to be the solvent

† Paper I in this series is the accompanying paper, Zahn et al. (1994*a*).

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exposure of hydrophobic amino acid residues, which are recognized by GroEL and thus screened against irreversible aggregation reactions. The complex is most stable when a maximal number of hydrophobic residues can be co-operatively bound by GroEL.

Since mature β-lactamase does not react with GroEL at 25°C, but a very strong interaction could be observed with the precursor, it appeared that a comparison of these two proteins might give information about the crucial features of substrate recognition by GroEL. Particularly, we wished to investigate whether there would be conditions under which the mature form would bind.

In the present paper we show that GroEL binds with high affinity to thermally unfolded mature β-lactamase. The reason for the recognition of thermally unfolded mature β-lactamase by GroEL is suggested to be both the destabilization of the native state of mature β-lactamase at high temperature and the increase in hydrophobic interaction with increasing temperature. These results are consistent with the suggested model of substrate recognition by GroEL.

2. Materials and Methods

Most materials and methods used are described in the accompanying paper (Zahn *et al.*, 1994*a*). Here, only additional methods and deviations from the previous methods are given.

(a) *Thermal folding experiments*

Thermal unfolding and refolding of β-lactamase was carried out in a folding buffer containing 100 mM potassium phosphate (pH 7·0), 100 mM urea, 100 mM ammonium sulfate, 0·01% Tween, 10 mM DTT, and the folding state was followed by measuring enzymatic activity or circular dichroism (CD). Thermal denaturation of GroEL was followed in folding buffer by measuring the fluorescence of aromatic amino acid residues, light scattering and anilinonaphthalene sulfonic acid (ANS†) fluorescence (see below).

(b) *Assay of β-lactamase activity*

Enzymatic activity measurements of β-lactamase were carried out as described previously (Laminet & Plückthun, 1989). During the assay 2 to 20 μl of the folding buffer were diluted in 1 ml assay solution containing nitrocefin, and β-lactamase activity was then determined by following the linear increase in absorption for 60 s. The nitrocefin assay contained no bovine serum albumin (BSA), in contrast to the original procedure, because BSA competes with β-lactamase for binding to GroEL at temperatures higher than 40°C. BSA was not found to be necessary to obtain linear kinetic traces for mature β-lactamase, while it is necessary for pre-β-lactamase.

(c) *Circular dichroism spectroscopy*

The decrease in secondary structure of β-lactamase (80 μg/ml) during thermal denaturation, was recorded by circular dichroism (CD) spectroscopy. Mean residue molar ellipticity $[\theta]_R$ was monitored at 222 nm with an Autodichrograph Mark IV (ISA Jobin Yvon) in a thermostated cell (Hellma) with 1 mm path length. The temperature was increased from 20 to 80°C at a linear rate of 0·5°C/min using a thermostat (RKS20, Lauda) with an automatic programmer (PM351, Lauda). Spectral changes were monitored continuously with 27 data points per min. The data were smoothed with the Macintosh program KaleidaGraph (window = 200 data points) and evaluated as described below.

(d) *Fluorescence spectroscopy and light scattering*

Measurements were carried out with a Shimadzu RF-5000 fluorescence spectrometer. During all experiments, the temperature was determined directly in the quartz cuvette and the buffer solution (2 ml) was stirred during the measurements.

Spectra of β-lactamase were recorded with an excitation wavelength of 295 nm. Both the slits for excitation and emission were set to 5 nm. Difference spectra were obtained by first measuring the respective buffer alone (with or without GroEL and urea) followed by addition of β-lactamase from a 10·2 μM stock solution. Spectra were recorded at 48°C after an equilibration time of 20 min.

Thermal denaturation of GroEL was followed by measuring light scattering at 400 nm and change in fluorescence emission at 310 nm and 330 nm after excitation at 280 nm and 295 nm, respectively. The measurement of ANS fluorescence was followed with an excitation wavelength of 400 nm and emission at 480 nm to obtain information about the hydrophobic properties of GroEL as a function of the temperature. The spectral bandwidth was 1·5 nm for excitation and 10 nm for emission. Data points were taken 20 min after each temperature increase.

(e) *Evaluation of folding and binding experiments*

Data points were fitted with the Macintosh program KaleidaGraph. In the thermal unfolding experiments of β-lactamase, the enzymatic activity or CD signal (y) was measured as a function of absolute temperature T. From these data, the midpoint of thermal unfolding T_m was determined from a 7-parameter fit (y_f, y_u, m_f, m_u, T_m, ΔH_m, ΔC_p) with eqn (1), as described by Pace (1990):

$$y = \frac{(y_f + m_f T) + (y_u + m_u T)\, e^{-\frac{\Delta H_m \frac{1-T}{T_m} - \Delta C_p\left(T_m - T + T \ln \frac{T}{T_m}\right)}{RT}}}{1 + e^{-\frac{\Delta H_m \frac{1-T}{T_m} - \Delta C_p\left(T_m - T + T \ln \frac{T}{T_m}\right)}{RT}}}, \quad (1)$$

where m_f and y_f, and m_u and y_u are the slope and the intercept of the pre- and post-transition baselines of the unfolding curve, respectively; ΔH_m is the enthalpy change at T_m; ΔC_p is the difference in heat capacity between the unfolded and folded protein; R is the gas constant. In the case of the thermal unfolding curves measured by enzymatic activity, m_u was set to zero. The fraction of native β-lactamase F_N was determined from enzymatic activity, while the fraction of totally unfolded β-lactamase F_U was obtained from CD measurements (see Zahn *et al.*, 1994*a*). The temperature dependence of the free energy change ΔG is given by a modified form of the Gibbs-Helmholtz equation:

† Abbreviations used: ANS, anilinonaphthalene sulfonic acid; scFv, single-chain antibody fragment.

$$\Delta G = \Delta H_m \frac{1-T}{T_m} - \Delta C_p\left(T_m - T + T\ln\frac{T}{T_m}\right). \quad (2)$$

The equilibrium constant K of a folding reaction was calculated using eqn (3):

$$K = e^{-\frac{\Delta G}{RT}}. \quad (3)$$

In the titration experiments of β-lactamase with GroEL, the concentration of remaining free β-lactamase [N] was measured by enzymatic activity as a function of total GroEL concentration [EL], under the assumption that every GroEL molecule has a defined number B of binding pockets EL_B, which bind independently to the substrate protein. The apparent equilibrium constant for dissociation K'_D was determined from a one-parameter fit using eqn (5), which was derived from the mass law (eqn (4)):

$$K'_D = \frac{([S] - [EL_BX])([EL_B] - [EL_BX])}{[EL_BX]} \quad (4)$$

$$[N] = [S] - \frac{[S] + K'_D + B[EL]}{2} - \frac{\sqrt{-4[S]B[EL] + (-[S] - K'_D - B[EL])^2}}{2}, \quad (5)$$

where [S] is the total concentration of β-lactamase; [N] is the concentration of free, enzymatically active β-lactamase; $[EL_BX]$ is the concentration of the complex between the GroEL-binding pocket EL_B ($= B[EL]$) and enzymatically inactive β-lactamase X; K'_D is the apparent dissociation constant, which contains both the dissociation constant of the complex and the folding equilibrium of β-lactamase.

3. Results

(a) *Thermal unfolding of mature β-lactamase*

Thermal unfolding of mature β-lactamase was measured by enzymatic activity and circular dichroism (CD). While enzymatic activity gives information about the loss of the integrity of the active site, potentially due to very small changes in the tertiary structure, the loss of secondary structure can be followed by determining the mean residue molar ellipticity $[\theta]_R$ at 222 nm. For the unfolding studies measures by enzymatic activity, β-lactamase was preincubated for 20 minutes in folding buffer at the temperature indicated and then (after dilution into the β-lactamase activity assay) the enzymatic activity was measured at the same temperature. There was a steady increase in activity up to 46°C and unfolding occurred with a transition midpoint of 51°C (Figure 1a,b). By measuring CD, an unfolding transition of mature β-lactamase was observed at 54°C. We propose to interpret these results as two different transitions with the following folding scheme:

$$U \rightleftharpoons H \rightleftharpoons N, \qquad \text{Scheme (I)}$$

where N is native β-lactamase, H is enzymatically inactive β-lactamase with native-like secondary structure (H stands for intermediate at neutral pH), and U is totally unfolded β-lactamase. We define two transitions, characterized by two equilibrium constants, $K_H^U = [H]/[U]$ and $K_N^H = [N]/[H]$. At temperatures lower than 56°C, the thermal unfolding of mature β-lactamase was reversible. Folding was irreversible only when the protein sample was incubated for long times (hours) at temperatures higher than 56°C. Therefore, the unfolding curves are thought to represent reversible thermodynamic equilibria. The free energy changes $\Delta G(25°C)$ of the N–H transition and the H–U transition were calculated with values of 9 kcal mol^{-1} and 4 kcal mol^{-1}, respectively.

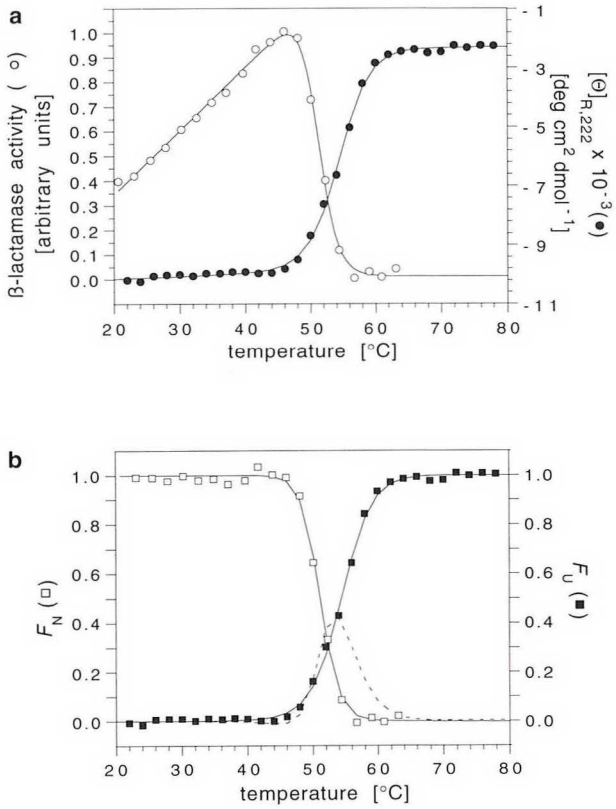

Figure 1. Thermal unfolding of mature β-lactamase. a, Enzymatic activity (○) of a β-lactamase solution (0·05 μM) was measured 20 min after raising the temperature by around 2·5°C to the temperature indicated. The temperature of the enzymatic activity assay was the same as in the folding buffer. Mean residue molar ellipticity $[\theta]_R$ (●) of β-lactamase (2·8 μM) in folding buffer was measured by far-UV CD at 222 nm. The $[\theta]_{R,222}$ of the folding buffer alone was constant in the measured temperature range (data not shown). Data from both thermal unfolding curves were fitted to eqn (1). b, Three-state equilibrium folding of β-lactamase. The fraction of β-lactamase in the native (F_N, □), unfolded (F_U, ■) and intermediate (F_H, ---) state are shown, as calculated from the denaturation curves in a.

(b) *Recognition of thermally unfolded mature β-lactamase by GroEL*

When the thermal unfolding of mature β-lactamase was carried out in the presence of two equivalents of GroEL, there was a shift in the transition midpoint of around 10°C to lower temperatures, as detected by enzymatic activity (Figure 2a). Bovine serum albumin (BSA) used as a control had no such effect. Therefore it is possible to set up an equilibrium system with

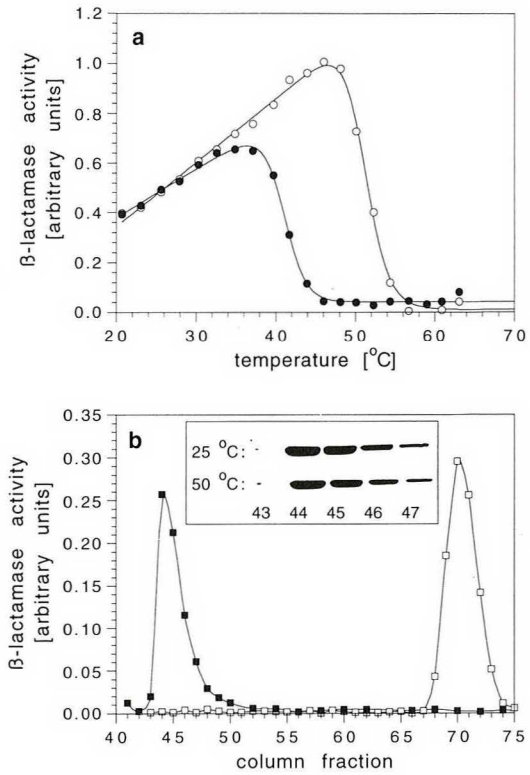

Figure 2. Recognition of thermally unfolded mature β-lactamase by GroEL. a, Thermal unfolding of β-lactamase. Enzymatic activity of a β-lactamase solution (0·05 μM) was measured 20 min after raising the temperature by around 2·5°C to the temperature indicated, in the presence (●) or absence (○) of GroEL (0·1 μM). The temperature of the enzymatic activity assay was the same as in the folding buffer. b, Gel filtration of mature β-lactamase and GroEL. Experiments were carried out with a temperature controlled Superdex™75 16/60 column (Pharmacia) equilibrated with folding buffer containing no Tween 20 at a flow rate of 0·5 ml/min. 200 μl folding buffer containing 15 μg β-lactamase (2·6 μM) and 417 μg GroEL (2·6 μM) was incubated for 20 min at 25°C (□) or at 50°C (■) and then loaded onto the gel filtration column equilibrated at the same temperature. After gel filtration, the β-lactamase activity of the column fractions (1 ml) was measured at 25°C and was confirmed by Wester blotting (data not shown). The GroEL containing fractions were determined by SDS-PAGE gel electrophoresis and Coomassie blue staining (insert).

GroEL and β-lactamase. By this interaction, the enzyme can form a stable complex far below its inactivation temperature of 51°C. The existence of temperature-dependent formation of a molecular complex between GroEL and mature β-lactamase was then examined directly using gel filtration. At 50°C, but not at 25°C, β-lactamase co-eluted with GroEL on the gel filtration column (Figure 2b). This binding was reversible without the addition of ATP, as was shown by the regain of β-lactamase activity at 25°C after gel filtration. Thus GroEL binds to a non-native state (X) of mature β-lactamase, as it does to precursor β-lactamase (pre-β-lactamase):

$$EL_BX \rightleftharpoons EL_B + N, \qquad \text{Scheme (II)}$$

where N is free native β-lactamase, EL_BX is the complex of the GroEL-binding pocket EL_B and non-native β-lactamase X. We define two equilibrium constants, $K_D = [X][EL_B]/[EL_BX]$ and $K'_D = [N][EL_B]/[EL_BX]$ where K'_D is an apparent dissociation constant, being the product of the dissociation constant K_D of EL_BX and the equilibrium constant K_N^X of the X–N transition.

K'_D of EL_BX was analyzed by titration experiments (Figure 3a). During this assay, the enzymatic activity of β-lactamase was measured after incubation at different GroEL concentrations and temperatures. The concentration of free β-lactamase was plotted as a function of total GroEL concentration from which K'_D could be estimated. Assuming a value for B of two binding sites per GroEL 14-mer, there is an exponential decrease in K'_D between 38°C and 50°C (Figure 3c). When measuring urea equilibrium curves of mature β-lactamase (in the absence of GroEL) between 38°C and 50°C (Figure 3b), we also found an exponential decrease of K_N^H with temperature (Figure 3c), which was comparable in magnitude to the decrease of K'_D (Figure 3c). Thus, although we do not know which state is stabilized in the complex with GroEL (H, U, or another state X) between 38°C and 50°C, the decrease in K_N^H seems to be sufficiently to at least partially explain the decrease in apparent dissociation constant K'_D of mature β-lactamase to GroEL at higher temperature.

The kinetic analysis of the GroEL-assisted disappearance of β-lactamase activity was consistent with a first order reaction (data not shown). The apparent association rate constant k'_a was independent of GroEL concentration (Table 1), indicating that unfolding of mature β-lactamase and not association of GroEL and β-lactamase is the rate-determining step.

(c) *Release of mature β-lactamase from GroEL*

GroEL-bound β-lactamase could be released by three different routes. The addition of ATP to the complex led to the regain of β-lactamase activity, indicating that GroEL-binding is ATP-reversible and thus "specific" (Figure 4a). Even when ATP was added at 50°C, folding was reversible without addition of GroES, as has been found for pre-β-lactamase (Laminet *et al.*, 1990).

Mature β-lactamase could also be released from GroEL by the addition of another competing protein, i.e. a single-chain antibody fragment (scFv; Glockshuber *et al.*, 1990). At 50°C and in the presence of 1·3 equivalents of scFv relative to GroEL and β-lactamase, 70% of the enzyme was replaced by the antibody (Figure 4b). A transient binding of an all-β-protein (a protein containing exclusively β-sheet secondary structure) has been recently shown by Schmidt & Buchner (1992) during refolding of antibody F_{ab} fragment (with its disulfides intact) at temperatures between 15°C and 25°C. The experiments with scFv at 50°C show that an all-β-protein can also be stably bound to GroEL, presumably because the disulfides become reduced

during the incubation (which contains free thiols). At temperatures higher than 40°C, we also observed stable binding between BSA and GroEL during the β-lactamase activity assay. Because of this finding we omitted BSA from our enzymatic activity buffer. BSA was only necessary to obtain linear traces for pre-β-lactamase, but not for mature β-lactamase.

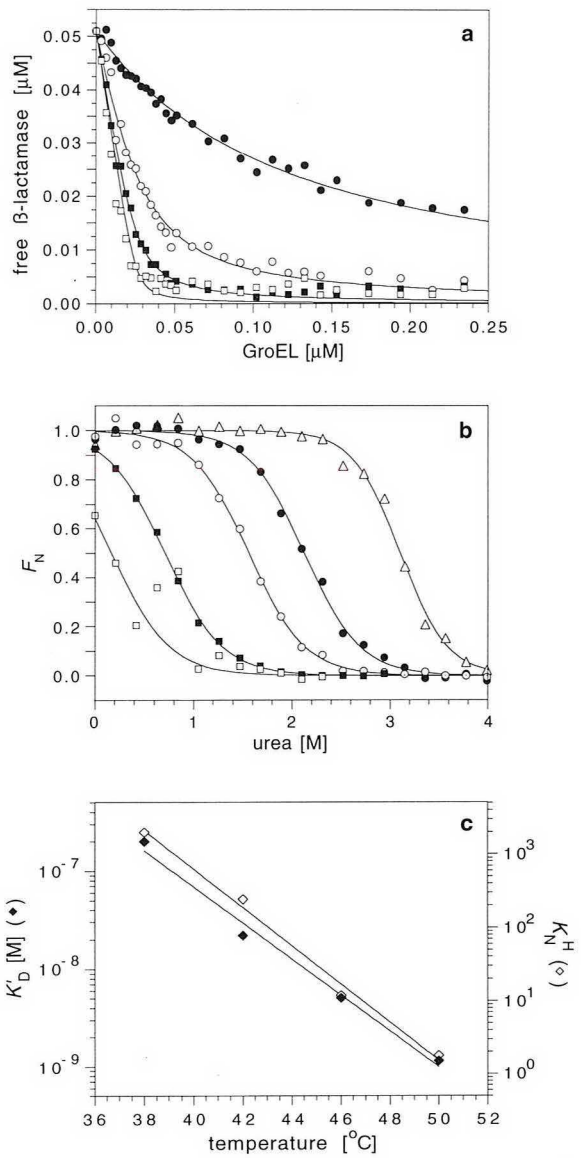

Figure 3. GroEL-assisted and urea induced unfolding of mature β-lactamase. a, Titration of β-lactamase (0·05 μM) with GroEL at 38°C (●), 42°C (○), 46°C (■) and 50°C (□). The concentration of free β-lactamase was determined by enzymatic activity after 15 h (at 38°C), 6 h (at 42°C), 2·3 h (at 46°C) or 0·7 h (at 50°C) of incubation at different total GroEL concentrations. The temperature of the enzymatic activity assay was the same as in the folding buffer. Data were fitted to eqn (5), assuming 2 molecules of β-lactamase binding per GroEL oligomer. b, Urea equilibrium unfolding of β-lactamase (0·5 μM) at 25°C (△), 38°C (●), 42°C (○), 46°C (■) and 50°C (□). β-lactamase activity was measured at 10°C after 18 h of incubation. Under these conditions, refolding during the assay is slow enough to be negligible. The fraction of native β-lactamase is shown. c, K'_D (◆) and K_N^H (◇) as a function of temperature, as determined from the equilibrium curves in a and in b with eqns (5) and (3), respectively.

As already mentioned, GroEL-bound mature β-lactamase could also be released by lowering the temperature of the protein solution. Between 10°C and 37°C, regain of β-lactamase activity was consistent with a first-order reaction (Figure 4c). At 10°C, there was an almost precise coincidence between the apparent dissociation rate constant k'_d of complex and the overall folding rate constant k_f of urea unfolded β-lactamase at the same temperature (Table 1). This is consistent with the finding that at 10°C the folding rate of urea unfolded β-lactamase was not influenced by GroEL (Zahn & Plückthun, 1992). In addition, ATP did not accelerate the regain in β-lactamase activity of GroEL-bound substrate protein at this temperature. The coincidence of rates at this temperature demonstrates that (1) mature β-lactamase is bound in an unfolded state to GroEL, (2) the folding reaction and not the release reaction is the rate-determining step and (3) the same transition-state must be crossed during folding of urea-unfolded and of GroEL-unfolded mature β-lactamase. At 25°C, k'_d was higher than at 10°C (Table 1), but about a factor of two slower than k_f of urea-unfolded β-lactamase in the absence of GroEL (Zahn *et al.*, 1994*a*). At 37°C, there was again a decrease of k'_d, which was more pronounced at higher concentrations of GroEL (Table 1). This is consistent with, at 37°C, (1) the net release reaction of GroEL-bound protein being at least partially rate limiting, (2) a transient rebinding of GroEL to β-lactamase during folding and (3) the rate limiting folding step of β-lactamase occurring in solution and not in the complex. We propose the following folding scheme:

$$EL_BX \underset{k_a}{\overset{k_d}{\rightleftharpoons}} EL_B + X \qquad \text{Scheme (III)}$$

$$X \xrightarrow{k_f} N,$$

where k_d is the dissociation rate constant of the complex EL_BX between the GroEL-binding pocket EL_B and enzymatically inactive β-lactamase X, k_a is the association rate constant and k_f is the folding rate constant of the rate limiting step of folding of GroEL-released (and urea-unfolded) β-lactamase to the enzymatically active state N.

Since the intrinsic folding reaction k_f, but not the overall reaction $k'_d = k_f\ K_D/[EL_B]$, gets faster at higher temperatures, there must be a decrease of the dissociation constant K_D between 10°C and 37°C. Thus, the reason for the stronger binding of mature β-lactamase at higher temperatures seems to be a destabilization of the native state and a temperature-dependent interaction between GroEL and the substrate protein.

Table 1
Kinetics of the apparent association and dissociation of mature β-lactamase and GroEL

T-shift† (°C)	β-Lactamase (μM)	GroEL‡ (μM)	ATP (mM)	Urea§ (mM)	Rate constant‖ (10^{-4} s^{-1} or $M^{-1}s^{-1}$)
A. *Association*					
25 → 45	0·05	0·06	—	100	13·6 ± 1·0
25 → 45	0·05	0·10	—	100	12·5 ± 1·1
25 → 45	0·05	0·60	—	100	15·7 ± 0·5
25 → 45	0·05	1·02	—	100	16·9 ± 1·0
B. *Dissociation*					
10 → 10¶	0·05	—	—	115	5·7 ± 1·0
50 → 10	0·05	0·10	—	115	7·2 ± 0·7
50 → 10	0·05	0·05	5	100	6·1 ± 0·6
50 → 25	0·26	0·26	—	100	28·6 ± 1·2
50 → 37	0·05	0·06	—	100	11·2 ± 0·9
50 → 37	0·05	0·60	—	100	0·8 ± 1·2

Conditions as in Materials and Methods (a, b). Data are analyzed according to first-order kinetics. Standard errors are given.

†Shift in temperature of the GroEL and β-lactamase containing folding buffer.

‡Concentration of GroEL 14-mer.

§Final urea concentration in the folding buffer.

‖Apparent rate constant for association (k'_a) or dissociation (k'_d).

¶Folding of 8 M urea unfolded β-lactamase at 10°C.

(d) *Thermal stability of GroEL*

In order to delineate the thermal stability and temperature-dependent structural changes of GroEL itself, we investigated several spectral parameters of GroEL as a function of temperature. There was a constant decrease, in the fluorescence of aromatic amino acid residues after excitation at 280 nm, with temperature (Figure 5a), which corresponds to the temperature-dependent fluorescence decrease of free tyrosine and tryptophan (Schmid, 1989). A similar decrease of GroEL fluorescence with temperature was also observed after excitation at 295 nm, with a break at around 64°C (Figure 5a). Because there is no tryptophan in the sequence of the GroEL (Hemmingsen *et al.*, 1988), the break in the tryptophan fluorescence results most probably from a change in the environment of GroEL-bound residual oligo- or polypeptides, which were not separated during the purification procedure (Hayer-Hartl & Hartl, 1993). At 64°C, there was also a steep increase in light scattering (Figure 5b) and in 1,8-anilinonaphthalene sulfonic acid (ANS) fluorescence of GroEL (Figure 5c), indicating an irreversible denaturation of GroEL at this temperature.

ANS has been proposed to bind to an exposed hydrophobic surface of GroEL in an ATP-reversible manner (Mendoza *et al.*, 1991) leading to a 20-fold increase in fluorescence at 22°C after excitation and emission at 400 nm and 480 nm, respectively. Unexpectedly, there was a strong continuous decrease in ANS fluorescence between 22°C and 58°C, while the fluorescence of ANS alone was almost constant in this temperature range (Figure 5c). Thus, the decrease in ANS fluorescence in the presence of GroEL indicates a change in interaction between the chaperone and ANS between 20°C and 58°C, reflecting a change in the interaction and/or the hydrophobic surface.

(e) *Tryptophan fluorescence of GroEL-bound mature β-lactamase*

The fluorescence of the four tryptophan residues of mature β-lactamase was observed at 48°C using an excitation wavelength of 295 nm (Figure 6).There was a decrease in fluorescence intensity and a shift of the fluorescence maximum from 342 to 352 nm after the native protein was unfolded in 8 M urea. The shift of the fluorescence maximum is consistent with a transfer of tryptophan residues from a hydrophobic environment in N to a hydrophilic environment in U. A decrease of fluorescence intensity after unfolding in guanidinium chloride and urea has also been observed for *Bacillus cereus* β-lactamase 569 (Goto & Fink, 1989) and precursor *E. coli* β-lactamase (Zahn & Plückthun, 1992), respectively. In the GroEL-bound state, there is an intermediate situation with a lowered fluorescence intensity relative to N but a fluorescence maximum at 342 nm. This would be consistent with an intermediate state of mature β-lactamase binding to GroEL, which might lead to dynamic quenching without concomitant shift of the fluorescence maximum. Alternatively, the tryptophan residues of a totally unfolded state of mature β-lactamase could be in direct contact with a hydrophobic GroEL-binding pocket.

4. Discussion

(a) *Three-state thermal unfolding of mature β-lactamase*

We propose to interpret the different thermal transition curves seen with the enzymatic activity and CD with a three-state model. This three-state model of thermal folding of mature β-lactamase described here (scheme (I)) is consistent with the

three-state urea equilibrium folding of mature β-lactamase and pre-β-lactamase examined in the accompanying article (Zahn *et al.*, 1994*a*). When comparing the equilibrium curves obtained from thermal and urea unfolding, there is a slightly more

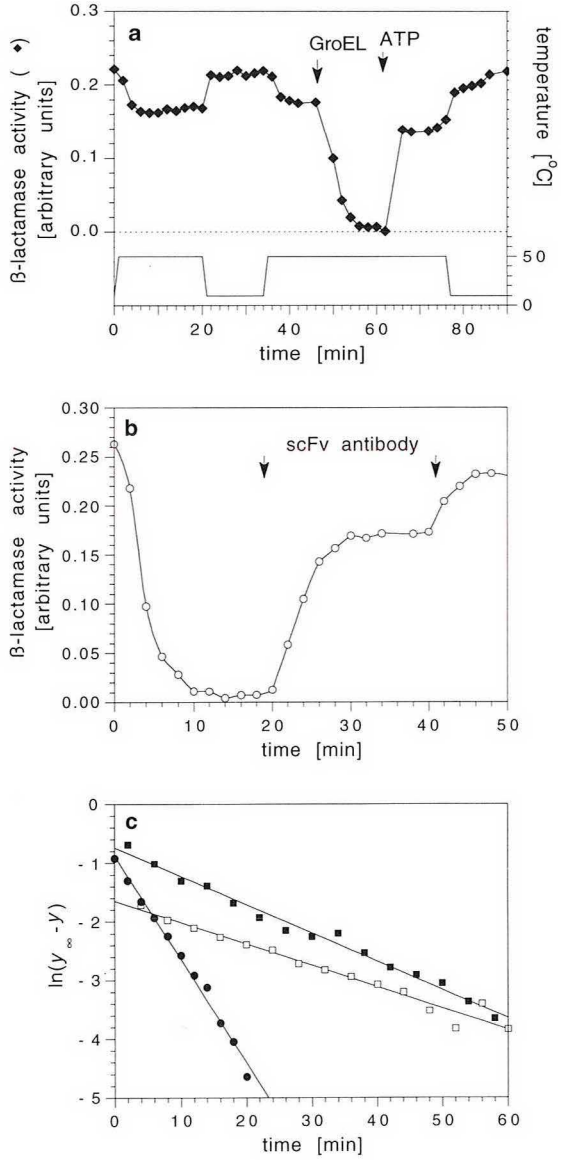

Figure 4. Release of GroEL-bound mature β-lactamase. a, The temperature of folding buffer containing β-lactamase (0·05 μM) was shifted between 10°C and 50°C. At the times indicated the β-lactamase activity was measured at 10°C (◆). At 48 min GroEL (0·05 μM) was added, and MgATP (5 mM) was added at 64 min. b, At $t = 0$, the temperature of folding buffer containing β-lactamase and GroEL (both 0·05 μM) was shifted from 25 to 50°C. At the times indicated the β-lactamase activity was measured at 10°C (○). At 19 min and 41 min 1·4 (left arrow) and 0·9 (right arrow) molar equivalents of single-chain antibody (scFv) McPC603 were added, respectively. c, First-order linear plots of the regain in enzymatic activity of GroEL-bound β-lactamase at 10°C (□), 25°C (●) and 37°C (■). y denotes the β-lactamase activity at time t, y_∞ at $t = \infty$. The temperature of the enzymatic activity assay was the same as of the folding buffer.

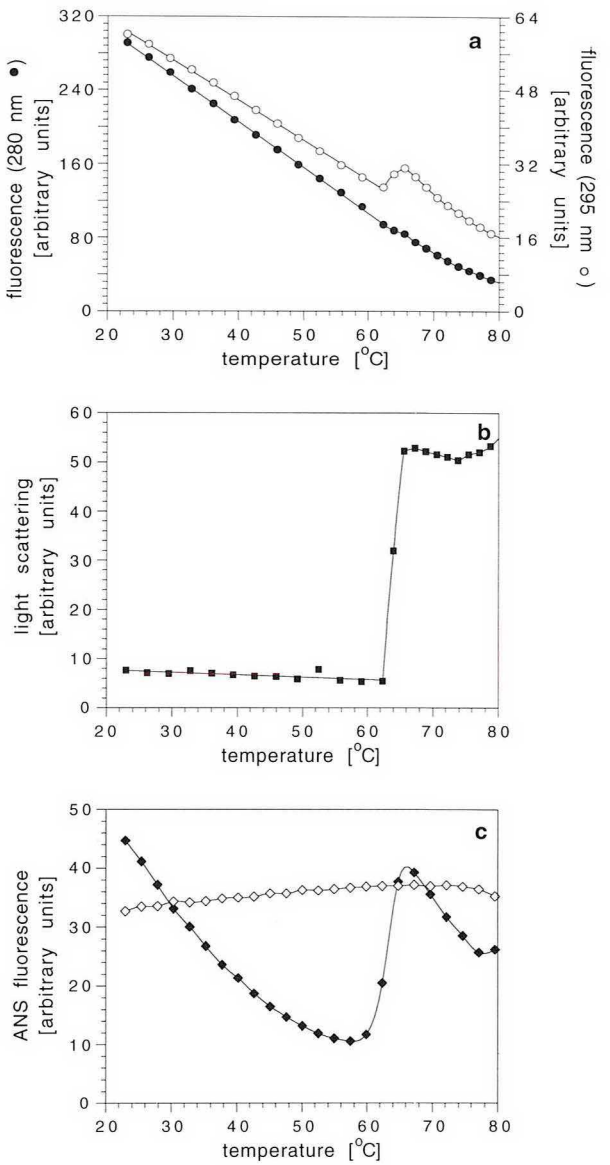

Figure 5. Thermal denaturation of GroEL. a, Fluorescence of GroEL (0·13 μM) at 310 nm (●) and 330 nm (○) after excitation at 280 nm and 295 nm, respectively. b, Light scattering of GroEL (0·13 μM) at 400 nm (■). c, Fluorescence of ANS (13 μM) in the presence of 0·13 μM GroEL (◆), and 260 μM ANS in the absence of GroEL (◇). Excitation and emission wavelength was at 400 nm and 480 nm, respectively. ANS fluorescence was measured in the absence of Tween 20, which is known to increase the fluorescence of ANS.

pronounced accumulation of the intermediate state (H) in the case of the urea experiments, while the two thermal transitions are more similar, leading to a narrow region of existence of H (Figure 1). However, there is correspondence in the free energy changes $\Delta G(H_2O, 25°C)$ with around 9 kcal mol^{-1} and 4 kcal mol^{-1} for the N–H transition and the H–U transition, respectively. Thus, the states H from thermal and urea equilibrium experiments seem to be similar with respect to enzymatic activity, secondary structure and environment of tryptophan residues. A thermodynamically stable intermediate has also been

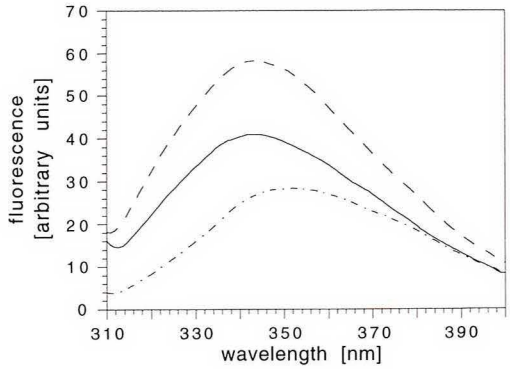

Figure 6. Tryptophan fluorescence of native (– – –), GroEL-bound (——) and urea unfolded (– · –) mature β-lactamase. Excitation was at 295 nm and the concentration of β-lactamase and GroEL was 0·1 μM. Difference spectra were recorded at 48°C.

observed for *Staphylococcus aureus* β-lactamase PC1 after unfolding by guanidinium chloride or urea (Robson & Pain, 1976*a*,*b*), and for *B. cereus* β-lactamase 569 after unfolding by low or high pH in the presence of high salt concentrations (Goto & Fink, 1989). These intermediates have been suggested to be molten globule states (Christensen & Pain, 1991), because they contain a sizeable hydrophobic core, a high content of secondary structure but no stable tertiary structure (Kuwajima, 1989, 1992). Thus, the accumulation of an intermediate state during equilibrium folding seems to be a general property of the sequence and structure-related class A β-lactamases (and their precursor proteins) and can apparently be induced by different denaturation procedures.

(b) *Recognition of thermal unfolded mature β-lactamase by GroEL*

A thermally denatured state of mature β-lactamase was found to bind to GroEL (Figure 2) and binding was reversible by ATP, by a competing substrate, or by temperature-shift (Figure 4). An ATP-reversible binding of GroEL (or a homolog Cpn60) to a thermally unfolded protein has also been reported for α-glucosidase (Höll-Neugebauer *et al.*, 1991), dihydrofolate reductase (Martin *et al.*, 1992), rhodanese (Mendoza *et al.*, 1992), P22 tailspike protein (Brunschier *et al.*, 1993), malate dehydrogenase (Hartman *et al.*, 1993), lactate dehydrogenase (Tagushi & Yoshida, 1993) and cyclophilin (Zahn *et al.*, 1994*b*). As for mature β-lactamase (data not shown), a non-native (enzymatically inactive) state of these proteins was protected against aggregation while they were in complex with GroEL. At elevated temperatures, the disappearance of enzymatic activity was either similar or increased in the presence of GroEL, as has been found for mature β-lactamase. This is consistent with the equilibrium of scheme (II) being towards the left hand side. The only exception was rhodanese, which showed an apparent stabilization of the native state of the enzyme in the presence of GroEL (and absence of ATP). The authors explained this with the binding of GroEL to a non-native, but enzymatically active state (N') of rhodanese, thus preventing the aggregation of N' to an enzymatically inactive state.

As has been observed for GroEL-bound P22 tailspike protein and cyclophilin (Brunschier *et al.*, 1993; Zahn *et al.*, 1994*b*), the mature β-lactamase was released from GroEL even without ATP, when the temperature of the protein solution was lowered to 30°C, consistent with the equilibrium between GroEL-bound and free protein of scheme (II) being towards the right hand side at this temperature. This temperature-dependent overall binding equilibrium K'_D of mature β-lactamase explains the lack in interaction between GroEL and this protein at 25°C or lower temperatures (Zahn *et al.*, 1994*a*). At least part of the decrease in the apparent dissociation constant K'_D of the complex between GroEL and mature β-lactamase with increasing temperature can be explained by the destabilization of the native state at higher temperatures (Figure 3).

(c) *Hydrophobic interaction*

Hydrophobic interactions show a characteristic temperature-dependence (Privalov & Gill, 1988; Creighton, 1993). The unfavorable transfer of a non-polar molecule from a non-polar liquid to water is usually seen as a result of an unfavorable change in entropy (ΔS_{tr}), thought to result from increased ordering of water molecules around the non-polar surface. Consequently, the hydrophobic interaction has the unusual property of decreasing in magnitude at lower temperatures, which is generally thought to result from the increasing tendency of water at lower temperatures to form hydrogen-bonded networks. This can be contrasted with other possible types of interactions between GroEL and substrate protein (i.e. hydrogen-bonds, van der Waals interactions, electrostatic interactions), which would decrease at higher temperatures.

There are different pieces of evidence for an increased interaction between GroEL and substrate protein with increasing temperature. (1) In the refolding experiments of GroEL-bound mature β-lactamase (Figure 4c) we observed a decrease of the apparent net dissociation rate constant k'_d (which encompasses all rebinding events) between 10°C and 37°C, although one would expect an accelerated folding rate at higher temperatures. Thus, while at 10°C the folding reaction of mature β-lactamase is rate-limiting, at 37°C the overall release reaction of the substrate protein from GroEL is the rate-determining step, indicating a decrease in K_D of the complex between GroEL and mature β-lactamase with increasing temperature, and this implies significant rebinding. (2) In the presence of two equivalents of GroEL with respect to mature β-lactamase, there was a decrease in the midpoint of the N–H transition from 51 to 41°C (Figure 2a). At

41°C, the free energy ΔG_H^N (25°C) of mature β-lactamase is still about 5 kcal mol^{-1}. In contrast, when mature β-lactamase was destabilized at 25°C with 2·5 M urea to a free energy of 3 kcal mol^{-1}, there was no binding to GroEL, although the chaperone itself is functional under these conditions (Horovitz *et al.*, 1993; Zahn *et al.*, 1994*a*). Thus, the affinity between GroEL and the protein must be higher at 41°C than at 25°C in 2·5 M urea. (3) The decrease in ANS-fluorescence of GroEL between 20°C and 58°C (Figure 5c) is most probably the result of a temperature-dependent competition between ANS and GroEL-bound oligo- or polypeptides. Although the purified GroEL was more than 98% homogenous on the SDS-polyacrylamide gel, there may be some residual bound peptides (Hayer-Hartl & Hartl, 1993), which cannot be separated from GroEL because of their high affinity to the chaperone.

Also consistent with a hydrophobic interaction is the intermediate tryptophan fluorescence maximum at 342 nm of GroEL-bound mature β-lactamase. This means either that some tryptophan residues of the bound protein are in a hydrophobic environment, or that all tryptophan residues of this state are in a partially hydrophobic environment. Interestingly, the same tryptophan maximum at around 340 to 345 nm has been observed for all GroEL-bound proteins studied so far, i.e. dihydrofolate reductase (Martin *et al.*, 1991), rhodanese (Martin *et al.*, 1991; Mendoza *et al.*, 1992), α-glucosidase (Höll-Neugebauer et al., 1991), β-lactamase precursor (Zahn & Plückthun, 1992), cyclophilin (Zahn & Plückthun, unpublished observation) and mature β-lactamase (Figure 6). The fluorescence maximum of these proteins in the native state is between 330 nm and 340 nm. Thus, the conformity in fluorescence maxima of the GroEL-bound substrates protein may reflect a hydrophobic GroEL binding pocket. This directly implies that fluorescence spectroscopy is not a suitable tool for making deductions about the conformational state of the bound protein.

In summary, the increased affinity between GroEL and mature β-lactamase at higher temperatures, suggested from the temperature dependence of scheme (III) and the spectroscopic properties of GroEL-bound polypeptides are consistent with hydrophobic interactions, between a hydrophobic GroEL-binding pocket and the solvent-exposed hydrophobic residues of an unfolded substrate protein, being the dominant force for substrate binding. This is consistent with the conclusions derived from the binding of hydrophobic peptides and the precursor protein (Zahn *et al.*, 1994*a*), and the binding of hydrophobic dyes (Mendoza *et al.*, 1991).

(d) *Conclusions*

In conclusion, the results of the experiments carried out in this investigation are compatible with a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL, suggested also from the experiments described in the accompanying article (Zahn *et al.*, 1994*a*). According to this model, there is a thermodynamic partitioning between the folding and the hydrophobic GroEL-binding of a protein. The binding of GroEL screens the solvent exposed hydrophobic residues and thus prevents irreversible aggregation reactions of unfolded substrate proteins. The temperature-dependence of this interaction makes this chaperone most effective at high temperatures, at which the danger of protein denaturation is enhanced *in vivo*.

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